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OXIDATION ENZYMES, THEIR PRODUCTION METHOD AND USES

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Abstract

Problem

To develop and provide new polyphenol oxidases having the optimum reaction pH on the alkaline side, microorganisms producing the polyphenol oxidases and their uses.

Mean to solve

Polyphenol oxidases produced by Stilbella, Sagenomella, and Stachylidium genus fungi. To provide methods for treatment of coloring materials, treatment of paper, pulp or fibers, treatment of microorganisms or viruses, washing, etc., using the above enzymes, deoxidizing agents and detergent compositions. To provide *Stilbella sp.* SD3101, *Sagenomella sp.* SD3102, and *Stachylidium sp.* SD3103.

Claims

1. Polyphenol oxidase having the following characteristics:

(1) action: it oxidizes polyphenol;

(2) optimum reaction pH: it has an optimum reaction pH of around 8.2;

(3) optimum reaction temperature: it has an optimum reaction temperature of around 50°C;

(4) molecular weight: its molecular weight measured by GFC analysis is about 76,000;

(5) isoelectric point: it has an isoelectric point of approximately 6.6, which has been measured by isoelectric point electrophoresis.

2. Polyphenol oxidase described in Claim 1, wherein Stilbella genus fungi produce said polyphenol oxidase.

3. Polyphenol oxidase described in Claim 2, wherein said Stilbella genus fungi are *Stilbella sp.* SD3101 (deposition number FERM P-15963).

4. Polyphenol oxidase having the following characteristics:

(1) action: it oxidizes polyphenol;

(2) optimum reaction pH: it has an optimum reaction pH of around 8.5;

(3) optimum reaction temperature: it has an optimum reaction temperature of around 50°C;

(4) molecular weight: its molecular weight measured by GFC analysis is about 33,000;

(5) isoelectric point: it has an isoelectric point of approximately 5.9, which has been measured by isoelectric point electrophoresis.

5. Polyphenol oxidase described in Claim 4, wherein Sagenomella genus fungi produce said polyphenol oxidase.

6. Polyphenol oxidase described in Claim 5, wherein said Sagenomella genus fungi are *Sagenomella sp.* SD3102 (deposition number FERM P-15964).

7. Polyphenol oxidase having the following characteristics:

(1) action: it oxidizes polyphenol;

(2) optimum reaction pH: it has an optimum reaction pH of around 8.2;

(3) optimum reaction temperature: it has an optimum reaction temperature of around 50°C;

(4) molecular weight: its molecular weight measured by GFC analysis is about 55,000;

(5) isoelectric point: it has an isoelectric point of approximately 7.0, which has been measured by isoelectric point electrophoresis.

8. Polyphenol oxidase described in Claim 7, wherein Stachylidium genus fungi produce said polyphenol oxidase.

9. Polyphenol oxidase described in Claim 8, wherein said Stachylidium genus fungi are *Stachylidium sp.* SD3103 (deposition number FERM P-15965).

10. A method for producing polyphenol oxidase described in Claim 1, characterized in that it is obtained by culturing of Stilbella genus fungi.

11. The method for producing polyphenol oxidase described in Claim 10, wherein said Stilbella genus fungi is *Stilbella sp.* SD3101 (deposition number FERM P-15963) or its variant.

12. A method for producing polyphenol oxidase described in Claim 4, characterized in that it is produced by culturing of Sagenomella genus fungi.

13. The method for producing polyphenol oxidase described in Claim 12, wherein said Sagenomella genus fungi are *Sagenomella sp.* SD3102 (deposition number FERM P-15964) or it's variant.

14. A method for producing polyphenol oxidase described in Claim 7, characterized in that it is produced by culturing of Stachylidium genus fungi.

15. The method for producing polyphenol oxidase described in Claim 14, wherein said Stachylidium genus fungi are *Stachylidium sp.* SD3103 (deposition number FERM P-15965) or it's variant.

16. *Stilbella sp.* SD3101 (deposition number FERM P-15963).

17. *Sagenomella sp.* SD3102 (deposition number FERM P-15964).

18. *Stachylidium sp.* SD3103 (deposition number FERM P-15965).

19. Detergent compositions containing a cleaning agent, detergent or surfactant and polyphenol oxidase described in any of Claims 1-9.

20. A method for bleaching colored materials, characterized in that the polyphenol oxidase described in any of Claims 1-9 is applied.

21. A method for treating microorganisms or viruses, characterized in that the polyphenol oxidase described in any of Claims 1-9 is applied.

22. A method for treating paper, pulp or fibers, characterized in that the polyphenol oxidase described in any of Claims 1-9 is applied.

23. A method for deoxidization, characterized in that the polyphenol oxidase described in any of Claims 1-9 is used.

Detailed explanation of the invention

[0001]

Technical field of the invention

The present invention relates to new polyphenol oxidases having the optimum reaction pH on the alkaline side, a method for producing such polyphenol oxidases from culturing of fungi, and their utilization method. More specifically, the present invention relates to new polyphenol oxidases having the optimum reaction pH near 8 on the alkaline side, microorganisms which produce the polyphenol oxidases, methods for producing the polyphenol oxidases, and their utilization methods in the fields of treatment, cleaning, etc., of colored materials, microorganisms or viruses, paper, pulp and fibers.

[0002]
Prior art

Until now, the polyphenol oxidation action by polyphenol oxidase or laccase produced by hyphomycetes such as basidiomycetes, deuteromycetes, etc., has been known. However, actual use of conventional polyphenol oxidation enzymes has been limited since the activity of these enzymes is notably low on the alkaline side. For example, the utilization of polyphenol oxidation enzymes for bleaching has been disclosed in WO 91-05839, EP 580707, DE 4008894, Japanese Kokai Patent Application No. Sho 64[1989]-60693, etc. However, the cleaning operation such as washing is carried out generally at an alkaline pH, and especially when oxidation and bleaching are simultaneously carried out in the presence of hydrogen peroxide, the cleaning operation at the alkaline pH is desired in order to accelerate the bleaching action of hydrogen peroxide. Therefore, actual utilization of conventional enzymes having the optimum reaction pH on the acidic side was difficult in the case where polyphenol oxidase was used for such uses.

[0003]

As natural coloring materials having polyphenol at the structure section, plant pigments such as flavonoid system, xanthene system, melanin system, and the like and lignin have been known, and polyphenol oxidases have bleaching action on said coloring materials. Further, polyphenol oxidase can be a reaction substrate even for dichlorophenol and trichlorophenol, which have problems in toxicity. Therefore, polyphenol oxidases are useful even in treatment of wastewater containing natural or non-natural materials as mentioned above. However, since conventional enzymes have the optimum reaction pH in the range of from acidic pH to neutral pH, the utilization of these enzymes at alkaline pH is extremely difficult, and this has been a main factor to make the industrial application scope of polyphenol oxidases narrow.

[0004]

On the other hand, among animal- and plant-origin polyphenol oxidases, ones having the optimum reaction pH at a high pH of 8 or higher have been known (Comp. Biochem. Physiol., 1992, 102B(4) 891-896: Zhongguo Nongye Huaxue Huizhi, 1991, 29(2), 177-185: Agric. Biol. Chem., 1991, 55(1), 13-17). However, it is difficult to produce these polyphenol oxidases stably and cheaply from tissues of animals and plants, and microorganism-origin polyphenol oxidases have been desired for industrial utilization.

[0005]

Problems to be solved by the invention

A problem to be solved by the present invention is to provide polyphenol oxidases having the optimum reaction pH on the alkaline side and a method for producing such polyphenol oxidases from culturing of fungi. Another problem to be solved by the present invention is to accomplish actual enzymatic oxidation in the alkaline zone by using polyphenol oxidases having the optimum reaction pH on the alkaline side and to contribute to expansion of application fields of polyphenol oxidases.

[0006]

Means to solve the problems

The present inventors had assiduously searched extracellular products, which catalyze the oxidation of polyphenol materials at alkaline pH, in a wide range of microorganisms, and it was found that strains of Stilbella genus, Sagenomella genus or Stachylidium genus, which are deuteromycetes, produce desired enzymes having the optimum reaction pH on the alkaline side near 8 as extracellular products, thus completing the present invention.

[0007]

Namely, the present invention provides the following.

[1] Polyphenol oxidase having the following characteristics:

(1) action: it oxidizes polyphenol;

(2) optimum reaction pH: it has an optimum reaction pH of around 8.2;

(3) optimum reaction temperature: it has an optimum reaction temperature of around 50°C;

(4) molecular weight: its molecular weight measured by GFC analysis is about 76,000;

(5) isoelectric point: it has an isoelectric point of approximately 6.6, which has been measured by isoelectric point electrophoresis.

[2] The polyphenol oxidase described in [1] is produced by Stilbella genus fungi.

[3] In polyphenol oxidase described in [2], the Stilbella genus fungi are *Stilbella sp.* SD3101 (deposition number FERM P-15963).

[0008]

[4] Polyphenol oxidase having the following characteristics:

(1) action: it oxidizes polyphenol;

(2) optimum reaction pH: it has an optimum reaction pH of around 8.5;

(3) optimum reaction temperature: it has an optimum reaction temperature of around 50°C;

(4) molecular weight: its molecular weight measured by GFC analysis is about 33,000;

(5) isoelectric point: it has an isoelectric point of approximately 5.9, which has been measured by isoelectric point electrophoresis.

[5] Polyphenol oxidase described in [4] is produced by Sagenomella genus fungi.

[6] In polyphenol oxidase described in [5], said Sagenomella genus fungi are *Sagenomella sp.* SD3102 (deposition number FERM P-15964).

[0009]

[7] Polyphenol oxidase having the following characteristics:

(1) action: it oxidizes polyphenol;

(2) optimum reaction pH: it has an optimum reaction pH of around 8.2;

(3) optimum reaction temperature: it has an optimum reaction temperature of around 50°C;

(4) molecular weight: its molecular weight measured by GFC analysis is about 55,000;

(5) isoelectric point: it has an isoelectric point of approximately 7.0, which has been measured by isoelectric point electrophoresis.

[8] Polyphenol oxidase described in [7] is produced by Stachylidium genus fungi.

[9] In polyphenol oxidase described in [8], said Stachylidium genus fungi are *Stachylidium sp.* SD3103 (deposition number FERM P-15965).

[0010]

[10] A method for producing polyphenol oxidase described in [1], characterized in that it is obtained by culturing of Stilbella genus fungi.

[11] The method for producing polyphenol oxidase described in [10], wherein said Stilbella genus fungi are *Stilbella sp.* SD3101 (deposition number FERM P-15963) or it's variant.

[12] A method for producing polyphenol oxidase described in [4], characterized in that it is produced by culturing of Sagenomella genus fungi.

[13] The method for producing polyphenol oxidase described in [12], wherein said Sagenomella genus fungi are *Sagenomella sp.* SD3102 (deposition number FERM P-15964) or it's variant.

[14] A method for producing polyphenol oxidase described in [7], characterized in that it is produced by culturing of Stachylidium genus fungi.

[15] The method for producing polyphenol oxidase described in [14], wherein said Stachylidium genus fungi are *Stachylidium sp.* SD3103 (deposition number FERM P-15965) or it's variant.

[0011]

[16] *Stilbella sp.* SD3101 (deposition number FERM P-1593).

[17] *Sagenomella sp.* SD3102 (deposition number FERM P-15964).

[18] *Stachylidium sp.* SD3103 (deposition number FERM P-15965).

[19] Detergent compositions containing a cleaning agent, detergent or surfactant and polyphenol oxidase described in any of [1]-[9].

[20] A method for bleaching colored materials, characterized in that polyphenol oxidase described in any of [1]-[9] is applied.

[21] A method for treating microorganisms or viruses, characterized in that polyphenol oxidase described in any of [1]-[9] is applied.

[22]. A method for treating paper, pulp or fibers, characterized in that polyphenol oxidase described in any of [1]-[9] is applied.

[23] A method for deoxidization, characterized in that polyphenol oxidase described in any of [1] to [9] is used.

[0012]

Next, the present invention will be explained in detail.

Preparation fungi

As strains of Stilbella genus to be used for obtaining polyphenol oxidase of the present invention, *Stilbella annulata*, *Stilbella bulbicola*, *Stilbella erythrocephala*, *Stilbella flavescens*, *Stilbella flavipes*, *Stilbella thermophila*, and *Stilbella sp.* are exemplified, but *Stilbella sp.* SD3101 (deposited as FERM P-15963 at Life Engineering Institute of Science and Technology, Agency of Industrial Science and Technology) is preferably used.

[0013]

Further, as strains of Sagenomella genus to be used for obtaining polyphenol oxidase of the present invention, *Sagenomella viride* and *Sagenomella sp.* are exemplified, but *Sagenomella sp.* SD3102 (deposited as FERM P-15964 at Life Engineering Institute of Science and Technology, Agency of Industrial Science and Technology) is preferably used. Further, as strains of Stachylidium genus to be used for obtaining polyphenol oxidase of the present invention, *Stachylidium bicolor*, *Stachylidium theobromae* and *Stachylidium sp.* are exemplified, but

Stachylidium sp. SD3103 (deposited as FERM P-15965 at Life Engineering Institute of Science and Technology, Agency of Industrial Science and Technology) is preferably used.

[0014]

On typical strains of the present invention, morphological observation such as tone, shape, and conidia of colonies, conidia-forming structure and the like was conducted after the strains were cultured on potato-carrot-agar medium and malt extract agar medium at 25°C for 14-60 days. As a result, hyphomycetes, which forms a white and mucilaginous colony similar to Acremonium, synnema, and small elliptical conidia with major axis of 3-5 μm, was named *Stilbella sp.* SD3101, similarly, hyphomycetes, which forms a white and mucilaginous colony similar to Acremonium, forms chain-form conidia without forming synnema, and forms elliptical to lemon-like small conidia with major axis of 2-4 μm, as *Sagenomella sp.* SD3102, furthermore, hyphomycetes, which forms a mucilaginous colony in dark brown to black, has a verticillated conidiophore and phialocondium, but does not form a sporodochium, as *Stachylidium sp.* SD3103.

[0015]

Preparation of enzyme

Polyphenol oxidase of the present invention is prepared from the culture of the aforementioned Stilbella, Sagenomella, or Stachylidium strain or its variant; it also can be prepared by utilization of gene manipulation of fungi. Namely, it can be produced even by the following method. Host cells, which have been transformed by using a suitable promoter and operator together with terminator DNA where DNA coding of the aforementioned polyphenol oxidase has an enzyme manifestation mechanism in the host organism and also a manifestation vector inserted in a DNA vector having a duplication initiation point for duplicating the vector in the host organism, or host cells which have been transformed by integrating a suitable promoter and operator together with terminator DNA where DNA coding of the aforementioned polyphenol oxidase has an enzyme manifestation mechanism in the host organism with host cell DNA are cultivated under conditions that polyphenol oxidase can be discovered and polyphenol oxidase is recovered from the medium.

[0016]

Further, polyphenol oxidases of the present invention may be produced by a protein engineering technique of alternating DNA of conventional polyphenol oxidase having the optimum reaction pH on the acidic side on the basis of the finding of the amino acid sequence of polyphenol oxidase obtained based on polyphenol oxidase-coding DNA sequence, etc. DNA

fragments, which code the polyphenol oxidases of the present invention, may be obtained by the conventional method of specifying the desired DNA fragment using an oligonucleotide synthesized based on the amino acid sequence of the present invention polyphenol oxidases or known polyphenol oxidases with cDNA or a genome library from strains of the present invention as a separation source, selecting a clone-revealing enzyme activity, or selecting a clone-producing protein which reacts with an antibody to the polyphenol oxidases.

[0017]

Commonly used synthetic culture medium or nutrient culture medium containing an organic carbon source and organic nitrogen source can be used for culturing to obtain polyphenol oxidases of the present invention. Further, it is desired to add a Cu^{2+} ion as a metal salt at a concentration of 0.001-10 mM and preferably 0.01-1 mM. The cultivation temperature is 10-60°C, preferably 20-40°C. Further, the proper cultivation time is 20-250 h and preferably 50-150 h. Secreted polyphenol oxidase can be recovered from the medium by a well-known method. The recovery procedure includes a series of procedures such as separation of cells from the medium by centrifugal separation, filtration or membrane separation and chromatography, for example, ion-exchange chromatography, etc. Further, membrane concentration using an ultrafiltration membrane is also effective.

[0018]

Enzyme properties

Polyphenol oxidases of the present invention carry out the oxidation reaction in a wide pH range of 5-11, preferably 6-10, more preferably 7-9 and have an optimum pH of near 8, and they have an advantage as a catalyst for oxidation reaction in the range from neutral to alkaline (Figures 1-3). Further, the optimum temperature in the reaction at pH 8 for 10 min is around 50°C (Figures 4-6); furthermore as the activity after being heated at various temperatures for 30 min, residual activity of almost 100% was shown in the temperature range of 50°C or lower (Figures 7-9). Furthermore, the residual activity after being treated in a buffer of various pH at 30°C for 30 min showed stability in a wide range of pH (Figure 10). The results assure the oxidation reaction in various solutions in a wide pH range from neutral to alkali at medium and low temperatures.

[0019]

Further, the polyphenol oxidases of the present invention can be used together with conventional enzymes having the optimum reaction pH on the acidic side. Namely, the polyphenol oxidase reaction can be carried out in a wide pH range from acidic to alkaline by

using a combination of the present invention polyphenol oxidases with conventionally known polyphenol oxidase having the optimum reaction pH on the acidic side. When enzymes are used for such purpose, the mixing ratio of the activity quantity of polyphenol oxidase having the optimum pH on the acidic side and the activity quantity of the present invention polyphenol oxidase is preferably 1:10 to 10:1, more preferably 1:3 to 3:1. The present invention polyphenol oxidases are useful even for accomplishing the polyphenol oxidase reaction in a wide pH zone.

[0020]

Method for measuring activity

In the present invention, the polyphenol oxidation activity was measured by carrying out the reaction in an aqueous solution containing 20 ppm syringaldazine and 100mM potassium phosphate buffer solution (pH 8.2) at 25°C and measuring the absorbance at 525 nm. Then, the activation quantity oxidizing 1 μmol syringaldazine for 1 min is defined as 1 unit (hereinafter abbreviated U).

[0021]

Utilization method of decomposition reaction and bleaching reaction

As a use of the new polyphenol oxidase having an optimum reaction zone at alkaline pH, for example, it can be used for bleaching. The utilization of polyphenol oxidases for bleaching is described in WO 91-05839, DE 400889, and Japanese Kokai Patent Application No. Sho 64[1989]-60693 etc. However, the cleaning operation such as washing is carried out generally at alkaline pH, and when oxidation and bleaching are carried out simultaneously in the presence of hydrogen peroxide, cleaning operation at alkaline pH is desired even for accelerating the bleaching action of hydrogen peroxide. Thereupon, in the application of polyphenol oxidases for this use, it was practically difficult to use conventional enzymes having the optimum reaction pH at acidic pH. The polyphenol oxidases of the present invention are useful for expanding uses of enzymes in alkali cleaning and alkali bleaching fields.

[0022]

At present, oxidation bleaching by hydrogen peroxide has been used in cleaning and washing. However, the bleaching power of hydrogen peroxide is not sufficient at 60°C or lower. Peracid precursor has been used together with hydrogen peroxide, but the bleaching powder at low temperature of 40°C or lower is not sufficient and a bleaching system having higher effect has been sought. Therefore, various enzymatic bleach acceleration methods have been proposed from the past. The oxidation bleaching at alkaline pH can be accelerated by existing polyphenol oxidase disclosed here with one or more substances having peroxidase action such as peroxidase,

lignin peroxidase, manganese peroxidase, and the like, and the usefulness of the present invention is clear.

[0023]

Hydrogen peroxide frequently used for oxidation bleaching is an expensive oxidizing agent, and hydrogen peroxide precursors, peracid precursors and peracids, which have been frequently used for detergents, are further expensive oxidizing agents. Further, it is possible to produce enzymatically hydrogen peroxide by using oxidase and its substrate, but this hydrogen peroxide generation system also may be considered as expensive oxidizing agents. The oxidation bleaching can be accelerated by combining the polyphenol oxidases disclosed here with one or more of air, enzymes, ozone, hydrogen peroxide, hydrogen peroxide precursors, peracid precursors, and peracids and using them. Therefore, the usefulness of the present invention is clear, which can accomplish oxidation bleaching by effectively using oxidizing agents.

[0024]

Of the oxidizing agents, hydrogen peroxide precursors dissolve in water to produce perhydroxyl ion. As such substances, there are, as monohydrate to tetrahydrate, perborate, percarbonate, perborax, sodium perpyrophosphate, perbenzoic acid, urea-H_2O_2 reaction product, melamine-H_2O_2 reaction product, citric acid perhydrate, and the like. Particularly preferred ones are perborate and percarbonate. Furthermore, hydrogen peroxide generation system by oxidase and its substrate can be used as hydrogen peroxide precursors. As examples of such oxidases, there are glucose oxidase, alcohol oxidase, glycerol oxidase, amine oxidase, amino acid oxidase, D-amino acid oxidase, aryl alcohol oxidase, aldehyde oxidase, galactose oxidase, sorbose oxidase, urate oxidase, xanthene oxidase, cholesterol oxidase, and the like, and particularly preferred ones are glucose oxidase and alcohol oxidase.

[0025]

Further, peracid precursors are organic compounds having a reactive acyl group or carboxylic acid ester, carboxylic acid anhydride, and acetates, and as such substances there are TAED (tetraacetylenediamine), TAMD (tetraacetylmethylenediamine), TAGU (tetraacetylglycoluril), DADHT (diacetyldioxohexahydrotriazine), SNOBS (sodium nonanoyloxybenzene sulfonate), ISONOBS (sodium isononanoyloxybenzene sulfonate), succinic anhydride, benzoic anhydride, phthalic anhydride, PAG (glucose pentaacetate), and xylose tetraacetate, and particularly preferred ones are TAED and SNOBS. Furthermore, as peracids, there are, for example, DPDDA (diperoxydodecanedioic acid), diperoxyisophthalic acid, magnesium monoperoxyphthalate hexahydrate, and NAPAA (nonylamidoperoxyadipic acid).

[0026]

The polyphenol oxidases of the present invention can be used together with various detergents, cleaning materials or surfactants. By this, detergents or cleaning material compositions mixed with the present invention polyphenol oxidases are provided. As typical examples of such detergents or cleaning material compositions, detergents or cleaning compositions containing, based on the weight of the detergents or cleaning compositions, 10-50 wt% surfactants, 0-50 wt% builders, 1-50 wt% alkali agents or inorganic electrolytes, and 0.1-10 wt% of at least one selected from the group of resoiling-preventing agents, enzymes, bleaching agents, fluorescent dyes, caking-preventing agents and oxidation-preventing agents may be exemplified.

[0027]

As surfactants, any of these surfactants which have been generally mixed as cleaning compositions can be used, namely they are soaps, for example, aliphatic sulfates such as linear or branched alkyl or alkenyl sulfate, amidosulfate, and alkyl or alkenyl sulfate having a linear or branched alkyl or alkenyl group and with single or multiple components of ethylene oxide, propylene oxide and butylenes oxide added, aliphatic sulfonates such as alkyl sulfonate, amide sulfonate, dialkyl sulfosuccinate, and various sulfonates of α-olefin, vinylidene olefin and inner olefin, aromatic sulfonates such as linear or branched alkylbenzene sulfonate, alkyl or alkenyl ether carbonates having a linear or branched alkyl or alkenyl group and with single or multiple components of ethylene oxide, propylene oxide and butylenes oxide added, amide α-sulfofatty acid salts or ester, amino acid type surfactants, phosphoric acid ester type surfactants such as alkyl or alkenyl acidic phosphoric acid ester and alkyl or alkenyl phosphate, betaine type amphoteric surfactants, alkyl or alkenyl ether or alcohol having a linear or branched alkyl or alkenyl group and with single or multiple components of ethylene oxide, propylene oxide and butylene oxide added, polyoxyethylene alkyl phenyl ether having a linear or branched alkyl or alkenyl group and with single or multiple components of ethylene oxide, propylene oxide and butylene oxide added, higher fatty acid alkanolamide or its alkylene oxide adduct, sucrose fatty acid ester, fatty acid glycerol monoester, alkyl or alkenylamine oxide, and tetraalkylammonium salt type cationic surfactants. In the case of anionic surfactants, sodium ion or potassium ion is preferred as a counterion. These surfactants are used singly or as a mixture of two or more.

[0028]

As builders and alkali agents or inorganic electrolytes, the following compounds can be used, namely: phosphates such as ortho-phosphoric acid salt, pyrophosphate, tripolyphosphate,

metaphosphate, hexametaphosphate and phytate; phosphonates such as ethane-1,1-diphosphonate and its derivatives, ethane hydroxy-1,1,2-triphosphorate ethane-1,2-dicarboxy-1,2-diphosphonate, methanehydroxyphosphonate, and the like; phosphonocarboxylic acid salts such as 2-phosphonobutane-1,2-dicarboxylate, 1-phosphonobutane-2,3,4-tricarboxylate, α-methylphosphonosuccinate, and the like; amino acid salts such as asparaginic acid, glutamic acid, and the like; aminopolyacetates such as nitrilotriacetate, ethylenediamine tetraacetate, diethylenetriamine pentaacetate, and the like; polymer electrolytes such as polyacrylic acid, polyitaconic acid, polymaleic acid, maleic anhydride copolymer, carboxymethylcellulose salt, and the like; non-dissociation polymers such as polyethylene glycol, polyvinyl alcohol, and the like; carboxymethylation compounds of diglycolic acid, oxydisuccinic acid, carboxymethyloxysuccinic acid, gluconic acid, lactic acid, tartaric acid, sucrose, lactose, and the like; carboxymethylation compound of pentaerythritol, carboxymethylation compound of gluconic acid; organic acid salts of benzene polycarboxylic acid, oxalic acid, malic acid, oxydisuccinic acid, gluconic acid, and the like; aluminosilicates such as zeolite; inorganic salts such as carbonate, sesquicarbonate, sulfate, metasilicate, and the like. Further, organic substances such as starch, urea etc., and inorganic compounds such as bentonite can be used. Furthermore, triethanolamine, monoethanolamine, triisopropanolamine, and the like can be used as organic alkali agents.

[0029]

The detergent compositions of the present invention contain surfactant and polyphenol oxidase of the present invention as constituent components as mentioned above, but if necessary the detergent compositions can further contain an amphoteric surfactant, bleaching agent such as perborate, percarbonate, etc., pigment, builder, resoiling preventing agent such as polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone, carboxymethylcellulose and the like, caking preventing agent, and other enzymes such as other oxidase, protease, lipase, amylase, cellulase, and the like.

[0030]

Any method can be used for mixing the detergent compositions of the present invention with enzymes such as polyphenol oxidase, but the mixing as a fine powder form is not preferred from the viewpoint of safety and hygiene of detergent users and workers in the detergent industry due to dusting during detergent handling, and it is preferred to use it in a solution state or shape into a dust-preventing state. The shaping may be carried out by round granulation, extruding granulation, fluidization granulation, centrifugal fluidization granulation or other

method, and the shape of the enzymes to be mixed in the detergent compositions of the present invention is not limited to the shaped one by the aforementioned method.

[0031]

As useful application fields for lignin removal and bleaching, strains of the present invention are inoculated in a portion of a pulp process to produce polyphenol oxidases of the present invention, or biopulping of directly adding a target enzyme product of the present invention to act on chips or coarsely crushed pulp, etc., and bio-bleaching may be exemplified. Even in these cases, the amount of chemical solutions for pH control can be reduced in the polyphenol oxidase having the optimum reaction pH on the alkaline side of the present invention as compared to conventional polyphenol oxidase having the optimum reaction pH on the acidic side so that reduction in cost is possible.

[0032]

Other utilization method

Biosensors utilizing polyphenol oxidases of the present invention can be used for monitoring aromatic compounds in various aqueous solutions and organic solvents having a pH range from neutral to alkaline by reflecting the characteristic of the present enzyme and are useful. Further, it is possible to carry out effective sterilization or inactivation of microorganisms or viruses in the pH zone from neutral to alkaline by utilizing the reactivity of phenoxy radicals generated from polyphenol oxidases of the present invention. Namely, it is possible to give rather strong germicidal property by phenoxy radicals enzymatically generated in addition to the germicidal property of polyphenol oxidase substrate itself. Moreover, when a sterilized material is contacted by a human body or taken into a human body or released into the environment, the substrate of polyphenol oxidase is changed to a less toxic material by oxidation and thus both of the sterilization property at the necessary point and the safety after that can be accomplished and the usefulness is high.

[0033]

As natural coloring materials having polyphenol in the structural part, plant pigments such as flavonoid system, xanthene system, melanin system, etc., and lignin have been known, and polyphenol oxidases have bleaching action on these natural substances. Further, polyphenol oxidases can be a reaction substrate for AOX such as dichlorophenol, trichlorophenol, and the like, which have a problem in toxicity. Therefore, the polyphenol oxidases of the present invention can be used even in treatment of wastewater containing such natural substances or non-natural substances.

[0034]

Further, the polyphenol oxidases of the present invention can be used for a deoxidization process or production of a deoxidization agent by utilizing the fact that the polyphenol oxidases act on phenol compounds, alkoxy group-containing aromatic compounds, halogenated phenol compounds, quinone compounds or aromatic amine compounds in an alkaline pH range to consume dissolved oxygen. In the deoxidization process or deoxidization agents, many natural or non-natural phenol compounds, alkoxy group-containing aromatic compounds, halogenated phenol compounds, quinone compounds or aromatic amine compounds can be utilized, and the dissolved oxygen concentration can be quickly lowered, thereby it is useful. Especially in the case of oxidation of a material group containing easily oxidizable polyphenols such as catechol, etc., the use of polyphenol oxidases at alkaline pH, which can advance simultaneously the automatic oxidation reaction at alkaline pH and the enzymatic and catalytic oxidation reaction of polyphenol, is extremely effective for deoxidization at high efficiency.

[0035]

Application examples

Next, the present invention is explained further in detail by showing typical examples. However, these are simple illustrations, and the present invention is not limited to these only.

[0036]

Application Example 1: Culture and concentration

Using 4 units of a 2-liter flask as a culture medium device, 2N NaOH was added to 500 mL of a medium containing 1% glucose, 0.2% NH_4Cl, 1.34% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4, 0.1% NaCl, 0.2% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.02 mM $CuSO_4$ to adjust the pH to 7.5 in each flask, and *Stilbella sp.* SD3101 (deposition number FERM P-15963) was inoculated and cultured at 27°C for 100 h under shaking. After cultivating, it was centrifuged at 4°C to obtain a bacteria-removed culture broth and it was concentrated as a fraction of molecular weight of 10,000 or higher by a Minitan ultrafiltration system (Millipore Co.) using a Minitan filter packet (CAT. No.: PTGCOMPO4, Millipore Co.).

[0037]

Application Example 2: Rough refining

The concentrated culture broth described in Application Example 1 was applied to the gap in the upper part of a column (ϕ60 mm, 330 cc) of a DEAE-Cellulofine A-800m (Seikagaku Kogyo Co.) equilibrated by 1.34% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4 and 0.1% NaCl and a

column washed with 400 mL of buffer having the same composition as the equilibrated one. Then, elution was carried out a total of 8 times by using 50 mL of 1.34% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4 and 0.3% NaCl for each time to obtain polyphenol oxidase activity in the fractions from the second elution to the fifth elution. These activity fractions were concentrated by the same ultrafiltration system as in Application Example 1. Further, after dialyzing against 200 ppm NH_4HCO_3, it was freeze-dried to obtain a roughly purified product as a freeze-dried product. The polyphenol oxidase activity of the freeze-dried product was 5 U/mg.

[0038]

Application Example 3: Culture and concentration, rough purification

The culturing and concentration of *Sagenomella sp.* SD3102 (deposition number FERM P-15964) were carried out in the same manner as in Application Example 1 to obtain an aqueous solution having polyphenol oxidase activity. Furthermore, the same rough purification as in Application Example 2 was conducted to obtain a freeze-dried product. The polyphenol oxidase activity of the freeze-dried product was 3.5 U/mg.

[0039]

Application Example 4: Culture and concentration, rough purification

The culturing and concentration of *Stachylidium sp.* SD3103 (deposition number FERM P-15965) were carried out in the same manner as in Application Example 1 to obtain an aqueous solution having polyphenol oxidase activity. Furthermore, the same rough purification as in Application Example 2 was conducted to obtain a freeze-dried product. The polyphenol oxidase activity of the freeze-dried product was 2.5 U/mg.

[0040]

Application Example 5: Measurement of isoelectric point

The active dyeing and isoelectric point of polyphenol oxidase activity samples obtained in Application Examples 2-4 were measured by isoelectric electrophoresis utilizing a Rotofoa system (BIO-RAD Co.). Farmalite (pH 2.5-5) (from Sigma Co.) was used as buffer. Furthermore, the active dyeing was carried out by immersing a gel plate after completing electrophoresis in an aqueous solution containing 50 ppm syringaldazine and 100mM potassium phosphate buffer solution (pH 8.2) and observing a reddish-violet band formed by the reaction with polyphenol oxidase. As a result, the isoelectric points of *Stilbella sp.* SD3101 (deposition number FERM P-15963), *Sagenomella sp.* SD3102 (deposition number FERM P-15964) and *Stachylidium sp.* SD3103 (deposition number FERM P-15965) were 6.6±0.5, 5.9±0.5, and 7.0±0.5, respectively.

[0041]

Application Example 6: Substrate specificity

The substrate specificity of the oxidation reaction was examined by using the roughly purified polyphenol oxidases described in Application Examples 2-4. The difference in the oxygen consumption rate at room temperature (25°C) between solutions containing 0.05mM substrate and 0.1M potassium phosphate buffer (pH 8.0) with and without addition of enzyme was measured by a manometer (YSI Model 5300 Biological Oxygen Manometer, Yellow Springs Instrument Co., Inc). Since the difference in oxygen consumption rate (Δ oxygen consumption rate) was highest in the case of using ascorbic acid as the substrate in this measurement, the value was set at 100 and the values relative to it are shown in Table 1. Furthermore, the same measurement was also carried out by using commercial ascorbic acid oxidase (*Cucumis sp.*, Funakoshi Co.) as a comparison and the result is also shown.

[0042]

Table 1

Substrate	Δ Oxygen consumption rate (relative value)			
	SD3101	SD3102	SD3103	Commercial enzyme
Syringaldazine	9.9	2.7	4.5	ND
o-Phenylenediamine	2.4	5.7	1.9	0.0
p-Phenylenediamine	18.4	6.1	13.1	0.0
Pyrocatechol	25.8	3.1	10.0	ND
Hydroquinone	1.5	2.3	0.4	0.0
Ferulic acid	46.4	3.9	23.8	0.0
Ascorbic acid	100	100	100	100
Bilirubin	30.3	1.9	8.2	0.0
Phenol	4.3	0.7	0.2	0.0
Aniline	1.4	0.2	0.2	0.0

[0043]

Application Example 7: Molecular weight

The molecular weight measurement was conducted by using GFC (gel filtration chromatography). The analysis, division and activity measurement of roughly purified polyphenol oxidases described in Application Examples 2-4 were carried out by HPLC utilizing a GFC column (Shodex PROTEINKW-802.5, duplex) equilibrated with 1.34% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4 and 1% NaCl at a flow rate of 1.0 mL/min and UV detector (280 nm). The activity peaks of polyphenol oxidases were eluted at the positions of molecular

weight of 76,000±5000, 33,000±5000, and 55,000±5000, respectively. Furthermore, MW-Marker (HPLC) of Oriental Industry Co. was used as the molecular weight marker.

[0044]

Application Example 8: Culture and rough purification in 5-liter flask

2N NaOH was added to 1 liter of a medium containing 1% glucose, 0.2% NH_4Cl, 1.34% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4, 0.1% NaCl, 0.2% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.02 mM $CuSO_4$ to adjust the pH to 7.5 in 2 units of a 5-liter flask, and *Stilbella sp.* SD3101 (deposition number FERM P-15963) was inoculated and cultured at 28°C for 5 days under shaking. After cultivating, it was centrifuged at 4°C to obtain 1.8 liters of a bacteria-removed culture broth. Then a portion of the culture broth was concentrated as a fraction of molecular weight of 10,000 or higher by a Minitan ultrafiltration system (Millipore Co.) using a Minitan filter packet (CAT. No.: PTGCOMPO4, Millipore Co.). It was further subjected to DEA-Cellulofine A-800m column chromatography, and the eluted active fraction was re-concentrated by the aforementioned Minitan ultrafiltration system as a fraction of molecular weight of 10,000 or higher. Further after dialyzing against 200 ppm NH_4HCO_3, it was freeze-dried to obtain a roughly purified product as a freeze-dried product. The polyphenol oxidase activity of the freeze-dried product was 8 U/mg.

[0045]

Further, by the same culture and rough purification operation, freeze-dried products of polyphenol oxidases, which produce *Sagenomella sp.* SD3102 (deposition number FERM P-15964) and *Stachylidium sp.* SD3103 (deposition number FERM P-15965), were obtained. The activity was 5 U/mg and 4.5 U/mg, respectively.

[0046]

Application Example 9: Treatment of soiled cloth by enzyme-containing detergent

An enzyme-mixed detergent was obtained by adding 20 mg of the freeze-dried product described in Application Example 8 to 1 g of an index detergent comprising 25 wt% sodium linear alkylbenzene sulfonate (LAS), 5 wt% polyoxyethylene lauryl ether, 15 wt% sodium tripolyphosphate, 6 wt% sodium silicate, 1 wt% carboxymethylcellulose sodium salt and 48 wt% Na_2SO_4, and the detergent which did not contain the freeze-dried product was referred to as non-enzyme-mixed detergent. Further, 0.2 mL 100 ppm Evans blue (Wako Pure Chemical Industry Co.) was added to the center of white cotton cloth (5 cm x 5 cm) to prepare a soiled cloth. Then, after one sheet of the soiled cloth and water 10 mL were put into a 500-mL beaker, 10 mg of the enzyme-mixed detergent or non-enzyme-mixed detergent were added and shaken

for 12 min to carry out washing treatment. The washed cloth was washed with water, air dried and its Y, y, and x values were measured by a color-difference meter (CR-200, Minolta Co.). Furthermore, the Z value was calculated by the equation [Z = (1-x-y)Y/y], and the improvement in whiteness was evaluated by the difference of the Z values (ΔZ value) before and after treatment. The enzyme addition effect (ΔΔZ value) was determined by comparing the improvement in whiteness when the enzyme-mixed detergent was used with the improvement in whiteness when the non-enzyme-mixed detergent was used, and the result is shown in Table 2.

[0047]

Table 2

Enzyme-producing microorganism	ΔΔZ value (enzyme addition effect)
Stilbella sp. SD3101	3.3
Sagenomella sp. SD3102	2.8
Stachylidium sp. SD3103	2.9

[0048]

Application Example 10: Mixing with conventional polyphenol oxidase

The activity measurement at various pH was carried out by using a mixture of the freeze-dried product described in Application Example 8 with polyphenol oxidase (*Rigidoporous zonalis* origin, obtained from Takara Co.) which has been marketed as a reagent. As shown in Figure 11, the oxidation reaction quickly advanced in a wide pH range of 4-10.

[0049]

Application Example 11: Deoxidization agent

The freeze-dried product, obtained from *Stilbella sp.* SD3101 (deposition number FERM P-15963 in Application Example 8, at a concentration of 10 ppm was acted on by 50 mM Na L-ascorbate as a phenolic compound at pH 9.0 and a temperature of 25°C, and the oxygen consumption rate was measured by a manometer. The dissolved oxygen concentration after 1 h from the reaction initiation was decreased to 0.4% so that it showed high deoxidization capability.

[0050]

Effect of the invention

As explained in detail above, new polyphenol oxidases having the optimum reaction pH on the alkaline side and methods for producing such polyphenol oxidases by culturing of fungi are provided by the present invention. Further, *Stilbella sp.* SD3101, *Sagenomella sp.* SD3102,

and *Stachylidium sp.* SD3103 of the present invention are useful for production of polyphenol oxidases of the present invention. Furthermore, methods for effective treatment of coloring materials, treatment of paper, pulp or fibers, treatment of microorganisms or viruses and cleaning treatment, etc., deoxidization agents and detergent compositions are provided by using polyphenol oxidases of the present invention.

Brief description of the figures

Figure 1 is a pH profile of polyphenol oxidase produced by SD3101.
Figure 2 is a pH profile of polyphenol oxidase produced by SD3102.
Figure 3 is a pH profile of polyphenol oxidase produced by SD3103.
Figure 4 is a temperature profile of polyphenol oxidase produced by SD3101.
Figure 5 is a temperature profile of polyphenol oxidase produced by SD3102.
Figure 6 is a temperature profile of polyphenol oxidase produced by SD3103.
Figure 7 shows temperature stability of polyphenol oxidase produced by SD3101.
Figure 8 shows temperature stability of polyphenol oxidase produced by SD3102.
Figure 9 shows temperature stability of polyphenol oxidase produced by SD3103.
Figure 10 is a graph showing the pH stability of polyphenol oxidase.
Figure 11 is a pH profile of polyphenol oxidase mixture.

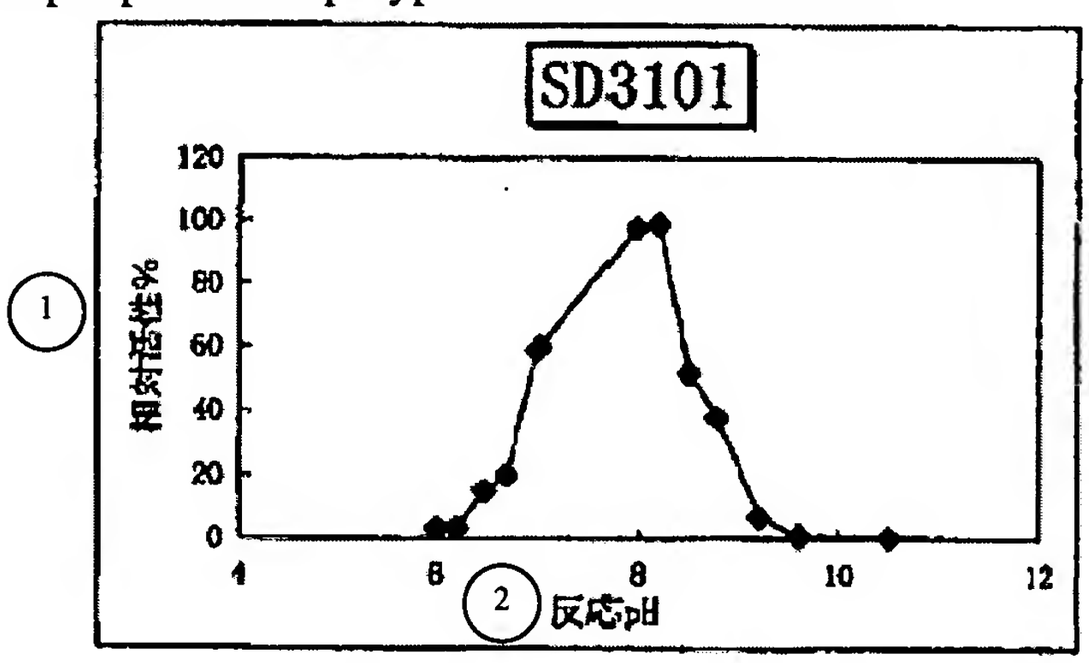

Figure 1

Key: 1 Relative activity %
2 Reaction pH

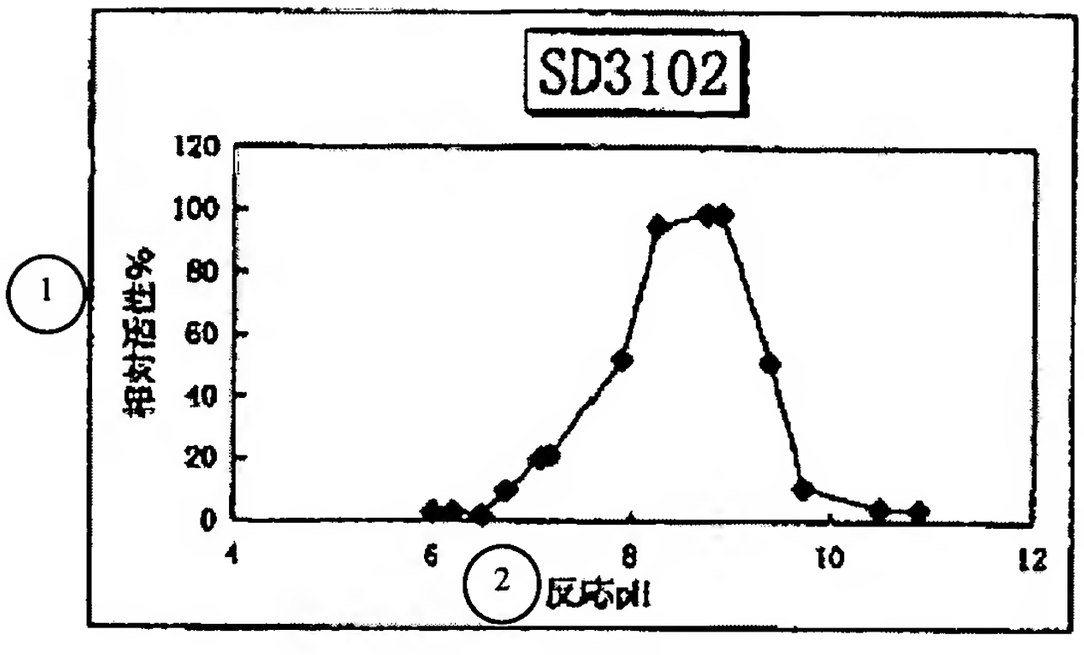

Figure 2

Key: 1 Relative activity %
2 Reaction pH

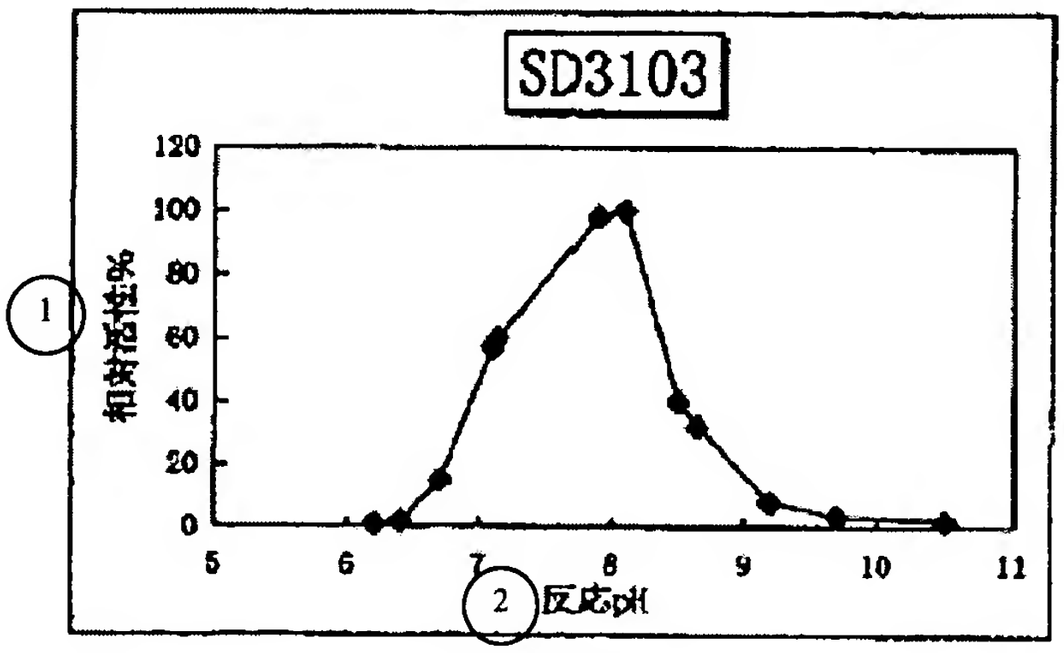

Figure 3

Key: 1 Relative activity %
2 Reaction pH

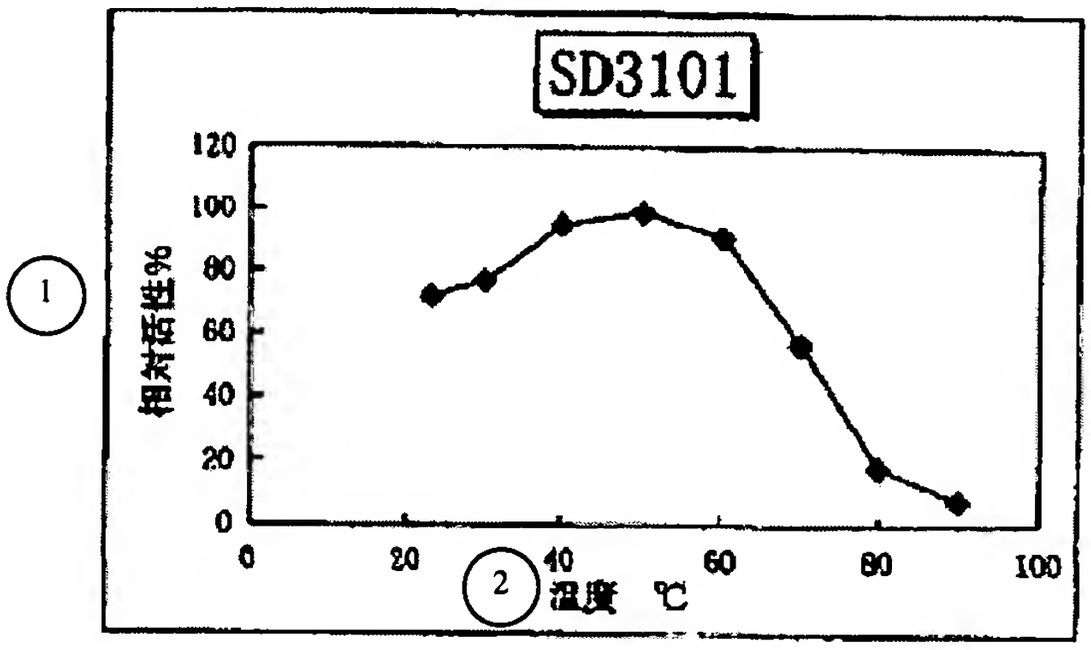

Figure 4

Key: 1 Relative activity %
2 Temperature °C

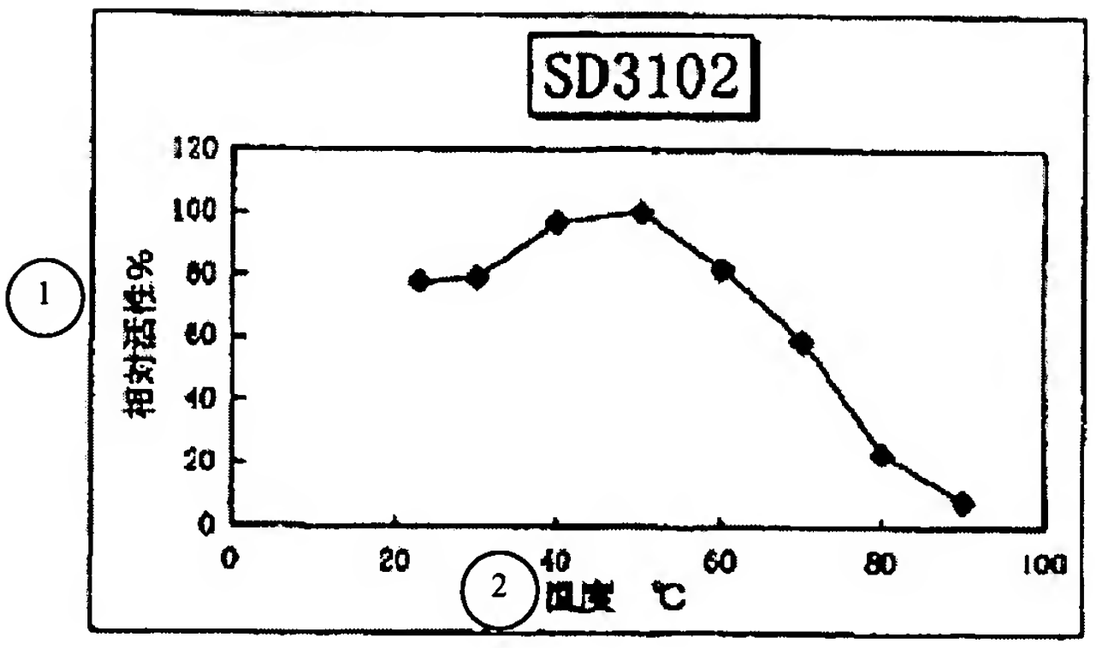

Figure 5

Key: 1 Relative activity %
2 Temperature °C

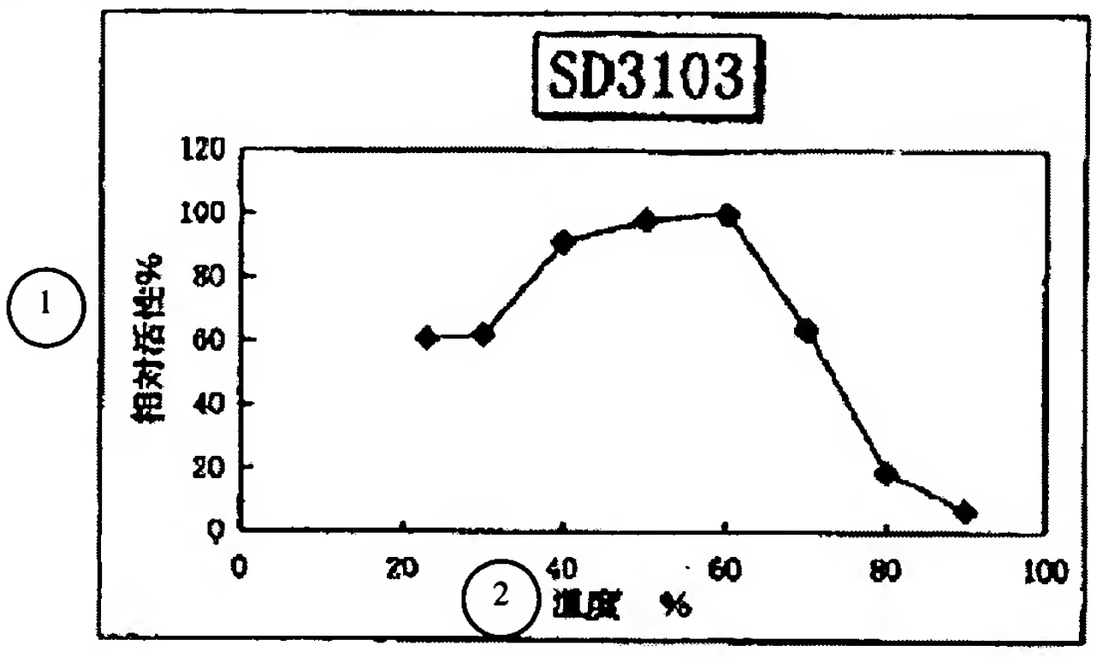

Figure 6

Key: 1 Relative activity %
2 Temperature % [sic; °C]

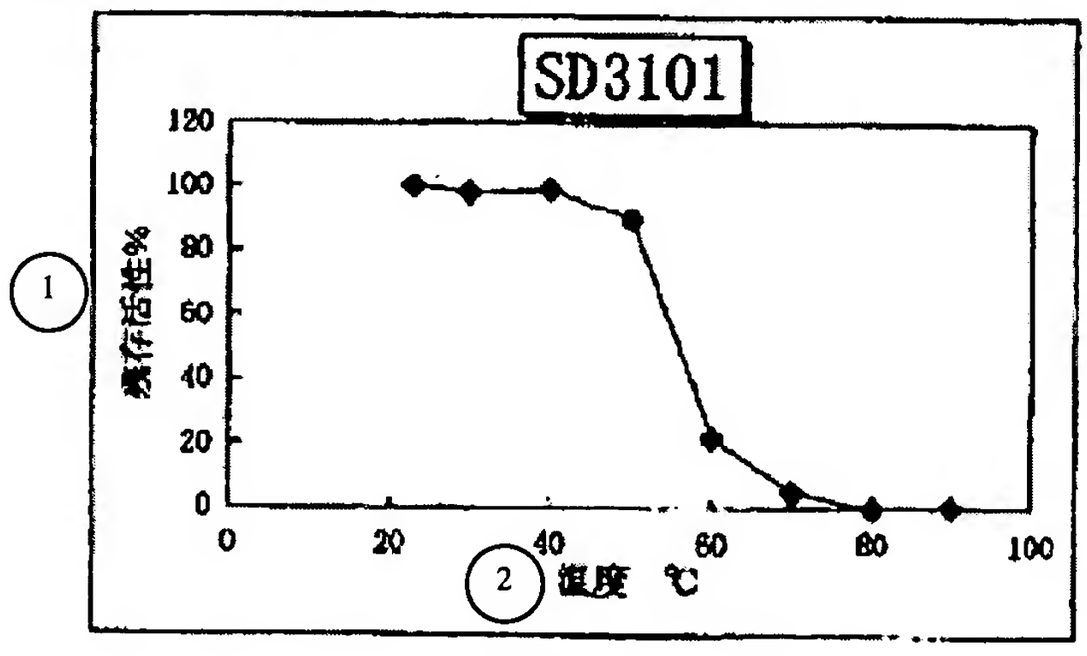

Figure 7

Key: 1 Residual activity %
2 Temperature °C

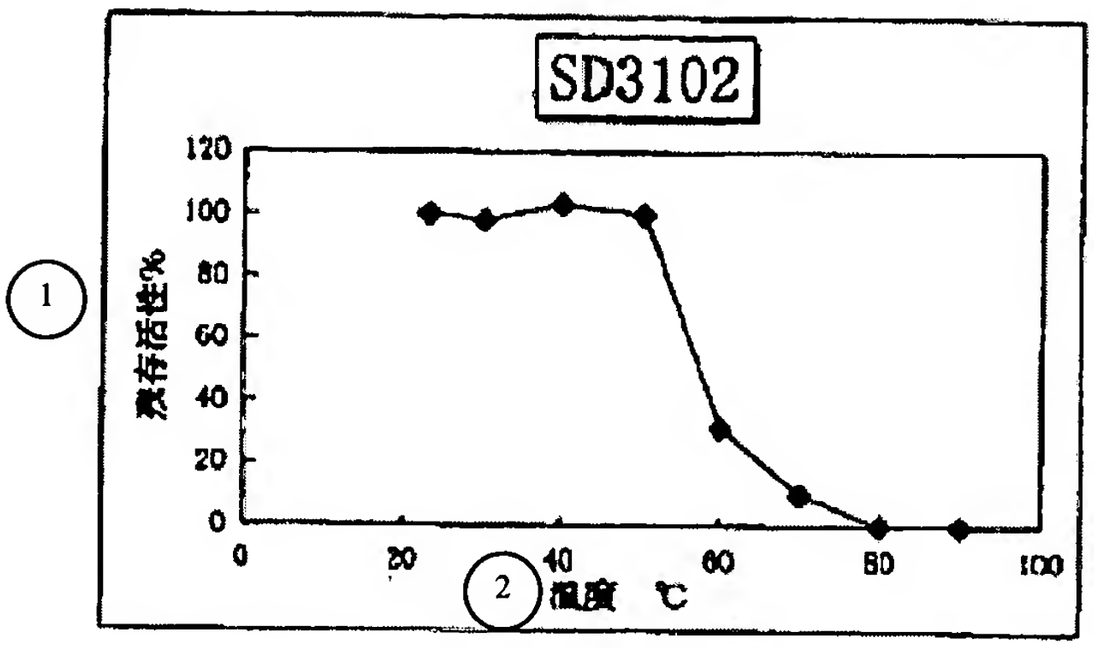

Figure 8

Key: 1 Residual activity %
2 Temperature °C

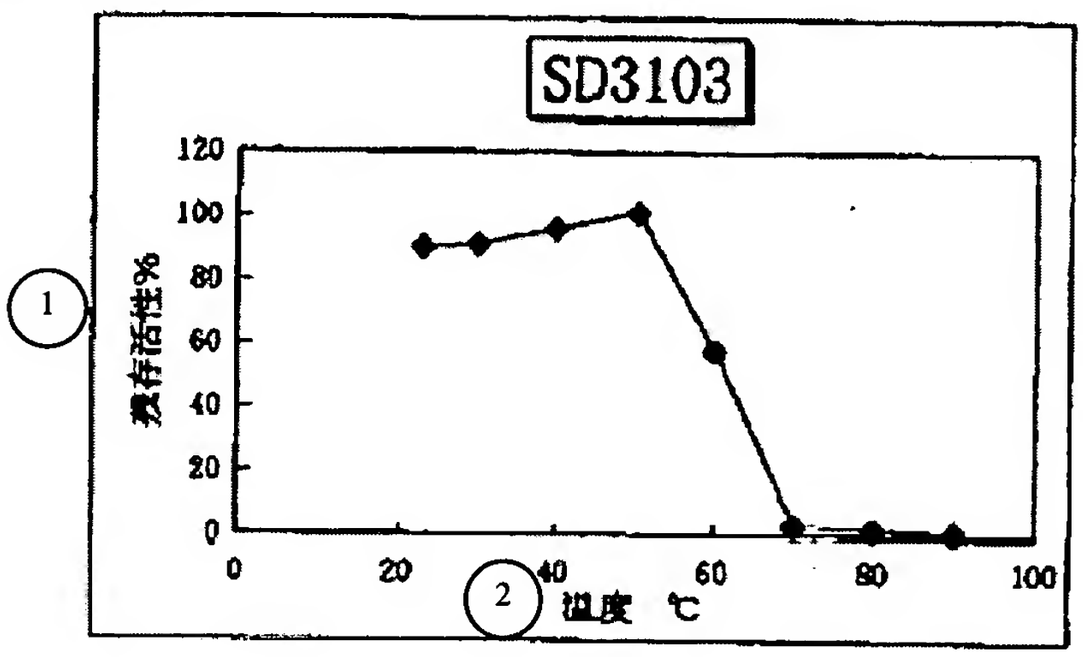

Figure 9

Key: 1 Residual activity %
2 Temperature °C

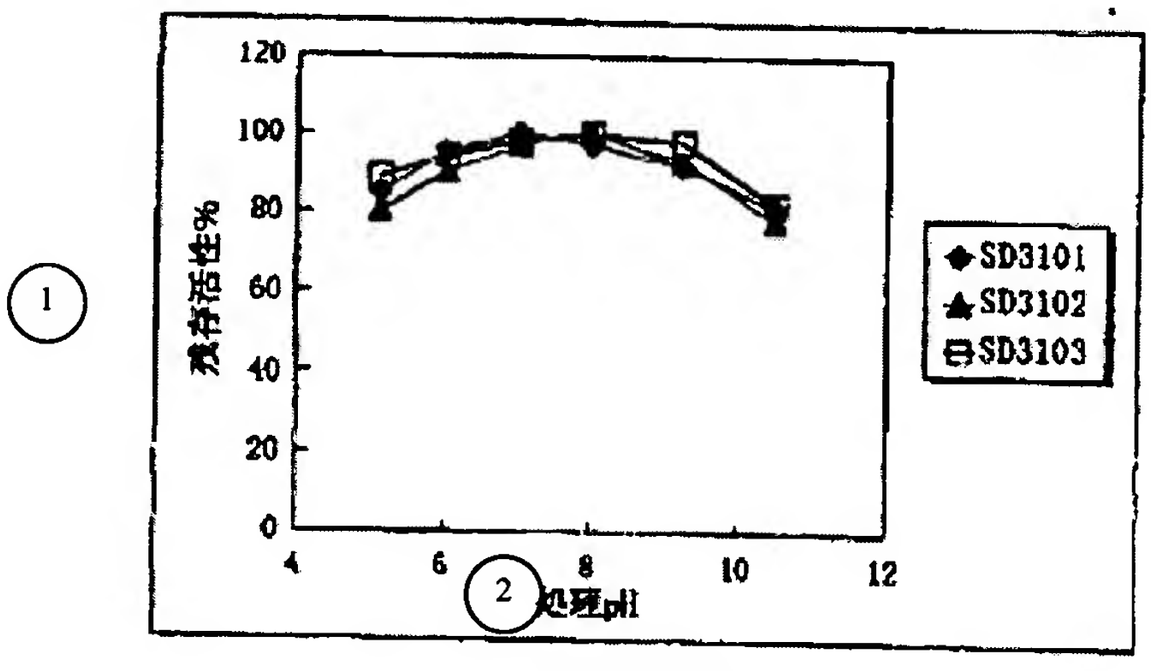

Figure 10

Key: 1 Residual activity %
2 Treatment pH

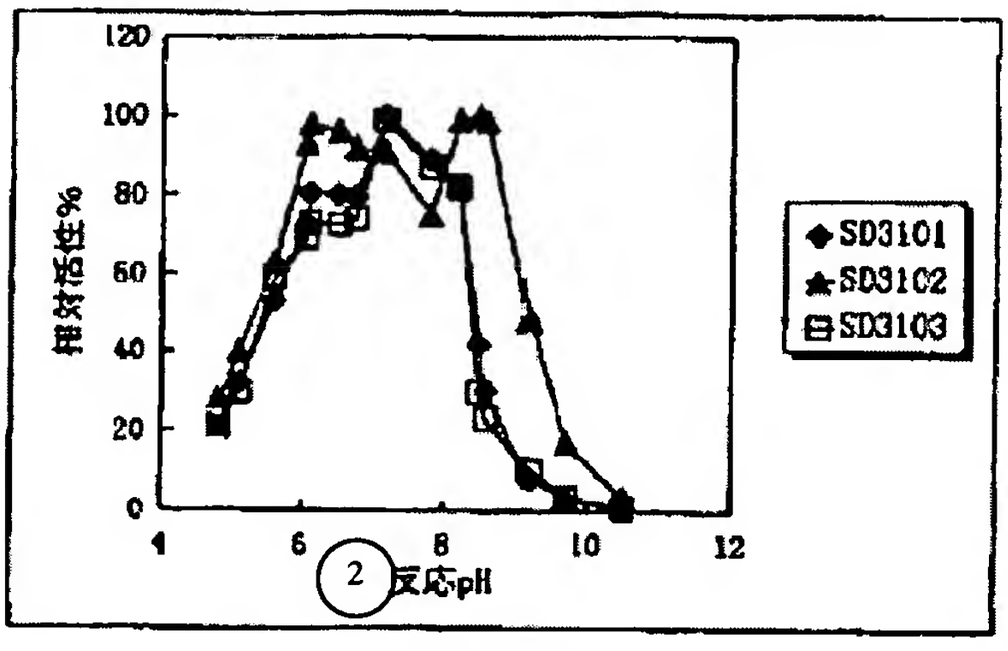

Figure 11

Key: 1 Relative activity %
2 Reaction pH